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Genomic Analysis of *blaKPC* Gene in *Klebsiella pneumoniae* Producing Carbapenemase

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Abstract: Carbapenem-resistant *Klebsiella pneumoniae* strains pose a significant threat to public health due to their ability to produce the *blaKPC* gene, conferring resistance to carbapenem antibiotics. Understanding the genomic diversity, evolutionary relationships and genetic complexity of *the blaKPC* variants is crucial for effective antimicrobial strategies. The study involved comprehensive genomic analysis of blaKPC gene sequences from 55 *K. pneumoniae* strains. The study involved indel haplotype diversity assessment, nucleotide diversity calculations and sequence conservation analysis. Additionally, the results were compared with larger dataset from a previous study. Indel haplotype diversity was Hd =0.879, nucleotide genetic variation was $\pi = 0.0395$ and the average number of differences in nucleotide was 37.446. Despite a smaller sample size, the study shows increased diversity of 8.9%. This study contributes valuable insights into blaKPC gene diversity, evolution and clinical relevance. Recommendations include tailored antibiotic selection, phylogenetic surveillance and global collaboration. Future studies should focus on functional characterization and clinical validation.

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I. INTRODUCTION

A class of Gram-negative bacteria called Enterobacteriaceae is present in the gut flora of humans. As members of the beta-lactam class of antibiotics, carbapenems work by preventing the formation of bacterial cell walls, which results in the death of bacteria. They are the most effective against both Gram-positive and Gram-negative bacteria, with a wide range of activity. Because of this, when patients develop life-threatening infections or are thought to be harboring resistant bacteria, they are frequently administered as last-line treatments or antibiotics of last resort. Regretfully, this class of life-saving medications is critically threatened by the rise of multidrug-resistant (MDR) bacteria (Shanmugam et al., 2013).

A growing global issue is carbapenem resistance in Enterobacteriaceae, specifically in *Klebsiella pneumoniae* and *Escherichia coli*. Carbapenemases are the most well-known enzymes that neutralize carbapenems, and several resistance mechanisms have been documented to evade the effectiveness of carbapenems. Among beta-lactamases, carbapenemases have the widest range. (Shanmugam et al., 2013).

Extended-spectrum β-lactamase (ESBLs) are a group of plasmid-mediated, diverse, complex, and rapidly evolving enzymes posing a major therapeutic challenge today in treating hospitalized and community-based patients. Infections due to ESBL producers range from uncomplicated urinary tract infections to life-threatening sepsis (Rawat & Nair, 2010).

blaKPC gene as one of the extended-spectrum Beta-lactamase encoding genes is a gene that encodes the production of the *Klebsiella pneumoniae* carbapenemase (KPC) enzyme. This enzyme is a type of beta-lactamase that can hydrolyze and inactivate carbapenem antibiotics, a class of broad-spectrum antibiotics used as a last resort for treating bacterial infections (Shanmugam et al., 2013).

The class-A carbapenemase of Klebsiella pneumoniae is a frequent mechanism of carbapenem resistance (KPC). The first isolate of K. pneumoniae that produced KPC was discovered in North Carolina in 2001. The enzyme is a beta-lactamase of the Amber class A (KPC-1). *Salmonella* spp., *Pseudomonas aeruginosa, Escherichia coli, Proteus mirabilis, Citrobacter freundii, Enterobacter* spp., *Serratia* spp., and *K. pneumoniae* and *Klebsiella oxytoca* have all been linked to KPCs. The transposable elements that surround the blaKPC genes, which encode KPCs, enable the genes to migrate back and forth between the bacterial chromosome and the transferable plasmid. All beta-lactam drugs, such as penicillin, cephalosporins, monobactams, and carbapenems, resist the KPC enzyme.(Shanmugam et al., 2013).

A. Background Information

Antimicrobial resistance has grown to be a global hazard to public health systems during the past few decades. Members of the Enterobacteriaceae family, especially

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Escherichia coli and Klebsiella spp., are among the bacteria that are most dangerous to human health due to their increasing resistance to antibiotics. The Enterobacteriaceae family of bacteria has developed a variety of antibiotic-resistant mechanisms, some of which are more diverse than those found in other families and include resistance to different groups of antibiotics. These advantages help to partially explain why these microorganisms are frequently responsible for antibiotic-resistant bacterial infections caused by multidrug-resistant *E. coli*, as these infections are spread through human feces and environmental sources. (Mendez, 2020).

In intensive care units in the United States, strains of *Enterobacter* spp. and *Klebsiell*a pneumoniae that are not sensitive to third-generation cephalosporins account for around 31% and 20% of infections, respectively. When *K. pneumoniae* acquires plasmid genes encoding for extended-spectrum β-lactamases (ESBLs), it usually results in resistance to third-generation cephalosporins. Additionally, these plasmids frequently carry other resistance genes (Paterson, 2006).

Since carbapenems are still effective against most Enterobacteriaceae, including those that produce ESBL, they are the recommended empirical treatment for severe infections caused by Enterobacteriaceae. Though uncommon, carbapenem resistance seems to be on the rise, with the advent of KPC-type carbapenemases being especially concerning. To stop ESBLs and other Enterobacteriaceae resistance from spreading farther over the globe, better antibiotic resistance stewardship is required (Paterson, 2006).

B. Problem Justification

Particularly for patients with impaired immune systems, extended-spectrum β-lactamase (ESBL) generating *Klebsiella pneumoniae* represents a significant risk of infection with elevated morbidity and death (Becker Laura, Fuchs Stephan, Pfeifer Yvonne, Semmler Torsten, Eckmanns Tim, Korr Gerit, Sissolak Dagmar, Friedrichs Michael, Zill Edith, Tung Mei-Lin, Dohle Christian, Kaase Martin, Gatermann Sören, Rüssmann Holger, Steglich Matthias, Haller Sebastian, 2018).

The rise of carbapenem-resistant Enterobacteriaceae (CRE) has generated challenges in clinical work due to the overuse of carbapenems. In addition to hydrolyzing carbapenems, KPC enzymes are the most significant class A carbapenemase enzymes. KPC-producing organism infections are linked to high death rates of up to 51%, which presents substantial challenges for clinical diagnosis and therapy (In et al., 2022).

The purpose of this work was to perform a thorough bioinformatics investigation of the blaKPC gene in *Klebsiella pneumoniae* that produces ESBL. The main goals of the study were to discover the genetic variants of blaKPC, comprehend how they are distributed across the many *Klebsiella pneumoniae* strains, and evaluate the possibility of horizontal gene transfer between pathogens. The results of this study may help understand the mechanisms underlying antibiotic resistance and guide the creation of potent defenses against the growing threat posed by germs that are resistant to several drugs.

C. Objectives of the Study

➢ *General Objective*

To look into the evolutionary relationships and genetic diversity among several species of carbapenemase that produce *Klebsiella pneumoniae*.

- *Specific Objectives*

✓ To clarify the evolutionary connections between different Klebsiella pneumoniae strains.
✓ To evaluate the genetic diversity and evolutionary trend of the carbapenemase-producing Klebsiella pneumoniae strains.

- *Conceptual Framework*

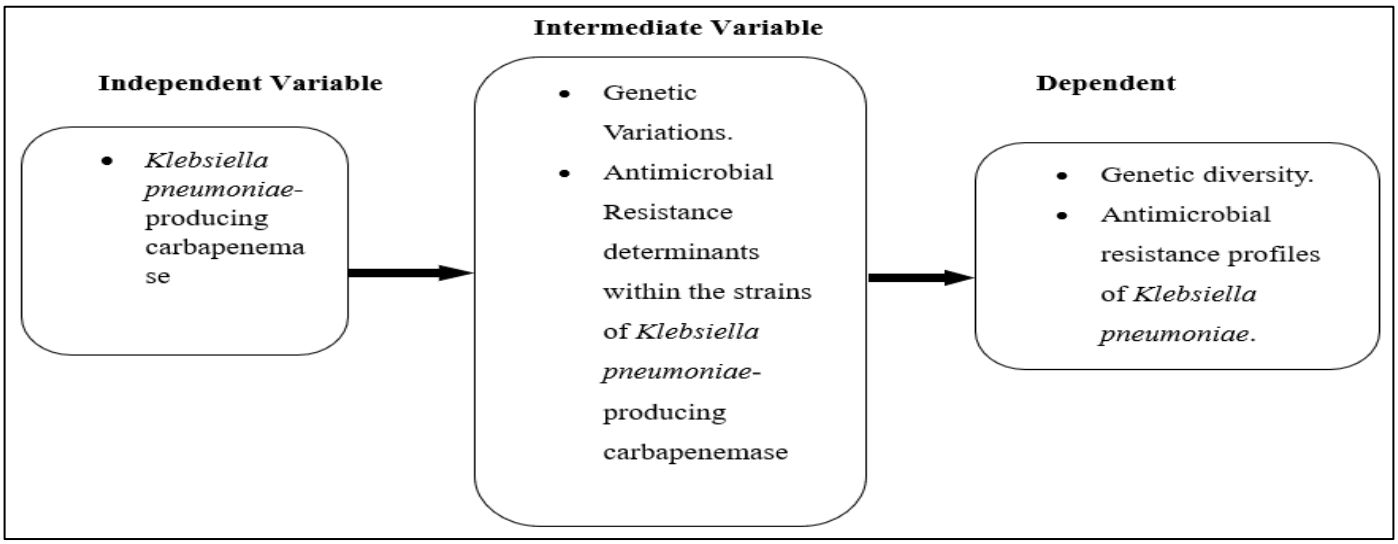

Fig 1 Conceptual Framework

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D. Hypothesis

- **Ho:** There isn't a significant genetic diversity and distinct evolutionary relationships between different species of *Klebsiella pneumoniae*-producing carbapenemase.
- **Ha**: There is a significant genetic diversity and distinct evolutionary relationships between different species of *Klebsiella pneumoniae*-producing carbapenemase.

II. LITERATURE REVIEW

Carl Friedlander isolated *Klebsiella pneumoniae* from the lungs of pneumonia victims and characterized it as an encapsulated bacillus in 1882. Gram-negative, encapsulated, non-motile bacteria called *Klebsiella pneumoniae* is present in the environment and has been linked to pneumonia in patients with diabetes mellitus and alcohol use disorders. The bacterium usually colonizes the gastrointestinal (GI) tract and oropharynx mucosal surfaces in humans. The bacteria can exhibit significant virulence and antibiotic resistance once it has entered the host. Presently, *K. pneumoniae* is thought to be the most frequent cause of hospital-acquired pneumonia in the US, and it is responsible for 3–8% of all nosocomial bacterial infections (Ashurst JV, 2023).

The rise in antibiotic resistance is threatening the lives of a great number of people worldwide. Antimicrobial resistance has led to an increase in the expense of treating and preventing infectious diseases. By changing the targets of medications, rendering them inactive, and turning on drug efflux pumps, bacteria can withstand the effects of antibiotics. Five million people are estimated to have died in 2019 from antibiotic-resistant causes, with 1.3 million of those deaths having a direct connection to antibiotic resistance (Moyo et al., 2023).

The rate of K. pneumoniae medication resistance worldwide has risen to 70%, and the mortality rate from infection-related causes has reached 40–70%. Both carbapenem-resistant Klebsiella pneumoniae (CRKP) and multiple-drug resistant (MDR) K. pneumoniae have become significant global public health issues in recent years.

100 of the 243 verified *Klebsiella* spp. carrying carbapenem resistance genes were found to have 43 percent of the carbapenemase-producing *Klebsiella pneumoniae* in a study conducted in South Africa. Of these isolates, 10 carried blaOXA-48-like, 17 carried blaKPC, and 73 carried blaNDM. (Ebomah & Okoh, 2020).

Disk diffusion susceptibility testing (DST) revealed that the overall prevalence of carbapenem resistance for both human and livestock isolates (41.7 percent) 80/192 was found in a study to determine *Klebsiella pneumoniae* carbapenemase in *Escherichia coli* isolated from humans and livestock in rural south-western Uganda. The DST-based resistance in the isolates from humans and livestock was the same (41.7 percent). According to the Modified Hodge Test (MHT), the prevalence of carbapenem resistance in isolates from humans and cattle was 5% (2/40) and 10% (4/40), respectively. 48.7% (95/192) of the isolates from both humans and livestock carried the KPC gene, which was more than the phenotypic expression (Tuhamize et al., 2023).

The CDC has recommended two steps to prevent antimicrobial resistance in a healthcare setting, which are the rational use of antimicrobials, regulations on the over-the-counter availability of antibiotics, improving hand hygiene, and improving infection prevention. The global threat of antimicrobial resistance has called for collaborative action to develop effective strategies for combating antimicrobials (Uchil et al., 2014). Further research is necessary to fully understand the global proliferation of blaKPC genes, even though several studies have examined their distribution. To limit the transmission of resistance genes, efforts for their prevention must take into account the patterns of worldwide dispersion (Forero-hurtado et al., 2023).

III. RESEARCH METHODOLOGY

The materials and techniques used in this research investigation are presented in this chapter. The purpose of these techniques and resources was to put the research study's hypothesis to the test.

➢ *Description of Study Area*

This research's study field included a thorough examination of genomic sequences from an international dataset. Secondary data from the primary sequence database National Center for Biotechnology (NCBI) was employed in the study.

The sequences studied in this study represented different geographical regions and settings, given the worldwide character of antibiotic resistance. This methodology enabled a comprehensive and varied analysis of the blaKPC gene in *Klebsiella pneumoniae* by offering perspectives on the global prevalence, distribution, and development of antibiotic resistance.

➢ *Research Design*

This study used a cross-sectional bioinformatics analysis as its research design. This approach was chosen to examine the genetic diversity, evolutionary tendencies, and prevalence of the blaKPC gene in Klebsiella pneumoniae using available genetic sequencing data from the National Center for Biotechnology (NCBI).

➢ *Data Collection*

Secondary data from the primary sequence database NCBI GenBank was used in the study. Obtaining *Klebsiella pneumoniae* nucleotide sequences with the blaKPC gene was a step in the data-gathering procedure.

The *K. pneumoniae* nucleotide sequences with the blaKPC gene were selected based on the sequences' completeness, quality, and relevance to the study goals.

- *Antibiotic Resistance Analysis:*

This will be done to identify resistant genes in the genomic sequences, it will be done by using Resfinder.

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➢ *Data Analysis*

The nucleotide sequences that were gathered were analyzed using bioinformatics methods and instruments. Included in the analysis were;

- *Sequence Alignment:*

This was used to identify conserved regions and mutations within the blaKPC gene from different strains of *Klebsiella pneumoniae*. During sequence alignment, ClustalW tools were used for multiple sequence alignment in MEGA.

- *Phylogenetic Analysis:*

Was used to determine the evolutionary relationships between blaKPC variants and their global distribution. The tools and software that were used were MEGA for phylogenetic tree inference.

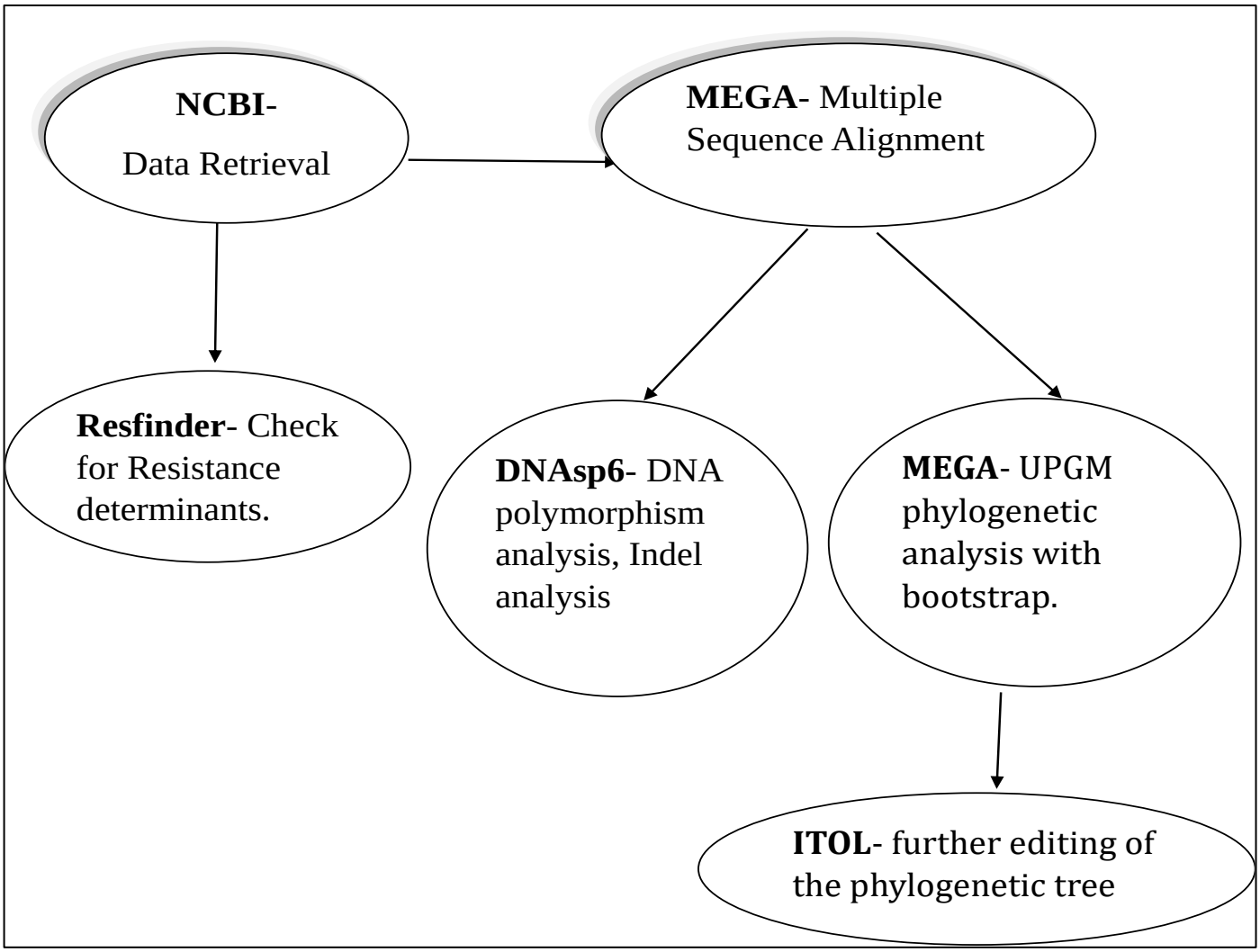

Fig 2 Showing the Data Collection and Analysis Workflow and all Bioinformatics Tools used in Data Analysis.

IV. RESULTS

A. DNA Polymorphism Analysis

It was done to understand how the genetic diversity was distributed across different strains of *Klebsiella pneumoniae-producing* carbapenemase.

Table 1 Showing the Number of Sequences and Selected Regions in DNA Polymorphism Analysis.

Number of Sequences	56
Number of Sequences Used	56
Selected Region	1-948
4.0 Number of Sites	948

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- *Pairwise Deletion Option:*

➢ *Sites with Alignment Gaps or Missing Data were Considered*

- *Analysis in Pairwise Comparisons*
  Gaps and missing information were excluded only in pairwise comparisons.

Table 2 Showing DNA polymorphism analysis with pairwise comparison of *Klebsiella pneumoniae* producing Carbapenemase (Rozas,J., Ferrer-Mata,A.,Sanchez-DelBarrio,J.C, Guirao-Rico, S., Librado,P., Ramos-Onsins S.E, Sanchez-Gracia, 2017).

Number of pairwise comparisons	1485
Average number of sites analyzed	774.46
The average number of differences	12.534
Nucleotide diversity	**0.0189**

- *Analysis at Individual Sites (Column by Column)*

Table 3 Showing DNA Polymorphisms Analysis at Individual Sites of *Klebsiella pneumoniae* Producing Carbapenemase Nucleotide Sequences

Number of sites analyzed	948
Number of polymorphic sites	312
The average number of differences	37.446
Nucleotide diversity, Pi	0.0395
Theta-W, per sequence	**84.91**
Theta-W, per site	0.08957

B. InDels (Insertion-Deletion) Polymorphism Analysis

➢ *The InDel Option used was Model Multiallelic while Analyzing Insertions and Deletions Polymorphism.*

Table 4 Showing InDels (Insertion- Deletion) Polymorphism Analysis Results

Total number of InDel sites analyzed	948
Total number of (InDel and non-InDel) sites analyzed	0 + 948
The total number of InDels events analyzed	1:27
Average InDel length event	93.667
Average InDel length	22.944
Number of InDel Haplotypes	24
InDel Haplotype Diversity	**0.873**
InDel Diversity, k(i):	**2.660**
Theta (per sequence) from I, Theta(i)-W:	**5.878**

C. Conserved Region Analysis

➢ *Dynamic Defined Parameters were used given the Observed S*

Table 5 Showing Conserved Region Analysis Results.

The net number of analyzed sites, L:	882
Number of variable/polymorphic sites, S:	260
Sequence conservation, C:	0.705
Minimum window Length, MWL:	70
Conservation threshold, CT:	0.8

➢ *Conserved Regions*

- *Region_1*

Table 6 Showing Region_1 Conserved Region

Start-End	Conservation	Homozygosity	P-value
1-526	0.938	0.995	0.000

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- *Region_2*

Table 7 Showing Region_2 Conserved Region

Start-End	Conservation	Homozygosity	P-value
819-948	0.835	0.987	0.0030

D. Phylogenetic Tree Analysis

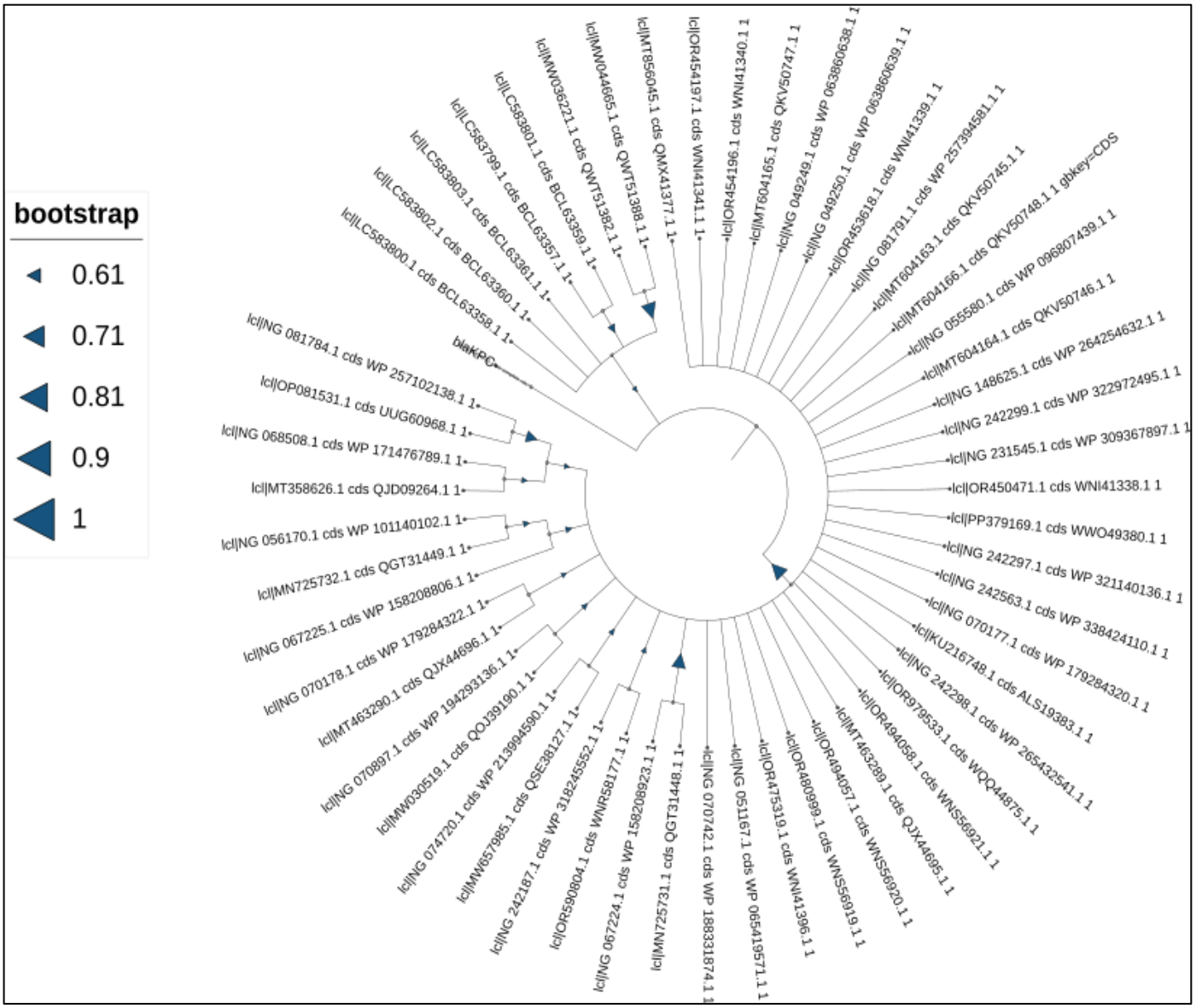

Fig 3 Showing UPGM Phylogenetic Tree of Klebsiella pneumoniae by using the Bootstrap method with bootstrap value 1000.

V. DISCUSSION

In the study, 55 sequences were used for their efficiency in capturing the diversity and patterns more effectively compared to smaller sample sizes and it can cover a diverse set of taxa or genetic variants that are relevant to the study, it also captures practical feasibility.

After obtaining the *K. pneumoniae* nucleotide sequences having blaKPC gene they were for antibiotic resistance determinants by using Resfinder. All the sequences showed high resistance to Penicillin, Cephalosporins, lincosamides, Macrolides, Tetracyclines, Beta-lactams, and Fluoroquinolones group of antibiotics which are the most commonly and only used antibiotics in the treatment of all bacterial infections. Beta-lactams and cephalosporin are used as last-line treatment of bacterial infections (Shanmugam et al., 2013). The resistance to cephalosporins and Beta-lactam antibiotics by *K. pneumoniae* could be caused by horizontal gene transfer from one bacterium to another.

During data analysis, multiple sequence alignment was first done to the Fasta format nucleotide sequences obtained from the National Centre for Biotechnology Information (NCBI). This was done using MEGA and ClustalW. Sequence alignment was done to identify regions of similarity and

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differences among the nucleotide sequences. These differences and similarities in the nucleotide sequences may indicate structural or evolutionary changes and relationships.

According to Ma & Liu, 2022 the distribution of resistance and virulence genes had little difference, but most strains had significant differences in the plasmid-encoded region. Most strains (31/36) carried the carbapenemase gene blaKPC-2, with no single nucleotide polymorphism in different strains. Extended-spectrum β-lactamase resistance genes, such as *blaCTX-M* and *blaSHV,* were found in the isolates, but no metallo-β-lactamases were detected (Ma & Liu, 2022), which shows that there has been a great evolution of most of the *blaKPC* genes such that there are gaps and single nucleotide polymorphism in different regions of the sequences obtained from the global dataset.

DNA polymorphism analysis was done using DnaSP6 to show the areas in the nucleotide sequence datasets that had variations. Determining the single nucleotide polymorphism could help in understanding the genetic diversity within the population. DNA polymorphism was done in two options: pairwise comparison and analysis at individual sites.

In comparison to Ma & Liu, 2022 whereby Prodigal V2.6.3 was used to predict all protein sequences of 36 isolates, orthofinderV2.5.2 calculated single-copy genes of the core genome according to protein sequences. Finally, Mafft V7.487 was used to compare sequences of 3827 core genes in 36 samples. Meanwhile, Gblocks Version 0.91b was used to shear the low-quality parts in the result. The phylogenetic tree was constructed by the maximum likelihood method from Version 2.1.10. In addition, the blaKPC gene of the isolated strain was analyzed by single nucleotide polymorphism (SNP) with BioEdit which showed no single nucleotide polymorphism meaning that there is a significant evolution among the strains of *blaKPC* gene since there was a significant single nucleotide polymorphism in the datasets (Ma & Liu, 2022).

Based on the DNA polymorphism analysis and InDel analysis in comparison to Spadar et al., 2023 on Large-scale genomic analysis of carbapenem-resistant hypervirulent strains there is a significant increased diversity of blaKPC genes in *Klebsiella pneumoniae* by 20.1% with a sample size of n=55.

In phylogenetic tree analysis, the method used was bootstrap method with bootstrap value of 1000 meaning that the nucleotide sequences were sampled randomly with each other by the bootstrap value during the creation of the UPGM phylogenetic tree in comparison to other studies whereby. The branches with values from 70% to 100% or 0.7 to 1 are more considered which show a high probability correct relation between or among the strains of *K. pneumoniae* compared to those lowered valued branches. From the phylogenetic tree only 14/55 nucleotide sequences show correct relation among each other meaning that the blaKPC strains in the *K. pneumoniae* have recently emerged due to low recombination, or due to horizontal gene transfer from other bacterial species.

VI. CONCLUSION

During the study, a substantial genetic diversity was revealed within the *K. pneumoniae* producing carbapenemase strains despite the smaller sample size which highlights the adaptive changes and evolutionary dynamics of the bacteria Klebsiella pneumoniae. Understanding the genetic variation is very crucial for effective antibiotic management and investigations between the specific variants and clinical outcomes. Based on the findings, further studies are warranted to investigate specific antibiotic regimens effective against *K. pneumoniae* strains producing carbapenemase, aiming to guide targeted treatment strategies.

RECOMMENDATIONS

From the study findings, phylogenetic surveillance of *K. pneumoniae* should be done continuously to identify high-risk clusters and to detect emerging hypervirulent or resistant strains. Collaboration and data sharing across institutions and countries should be done to influence guidelines for infection control and antimicrobial stewardship. Tailored treatment should be considered for individualized antibiotic regimens based on blaKPC gene variants. Resistance should be monitored regularly to assess resistance patterns to adapt treatment strategies.

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